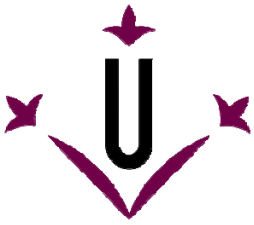

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INNATE AND LEARNED PREY-SEARCHING BEHAVIOR IN A GENERALIST PREDATOR

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Abstract- Early colonization by *Zyginidia scutellaris* leafhoppers might be a key factor in the attraction and settling of generalist predators such as *Orius spp*. in maize fields. In this paper we aimed to determine whether our observations of early season increases in field populations of *Orius spp*. reflect a specific attraction to *Z. scutellaris*-induced maize volatiles, and how the responses of *Orius* predators to herbivore-induced volatiles (HIPVs) might be affected by previous prey experiences. We therefore examined the innate and learned preferences of *Orius majusculus* towards volatiles from maize plants attacked by three potential herbivores with different feeding strategies, leafhopper *Z. scutellaris* (mesophyll feeder), lepidopteran *Spodoptera littoralis* (chewer) and leafhopper *Dalbulus maidis* (phloem feeder). In addition, we examined the volatile profiles emitted by maize plants infested by the three herbivores. Our results show that predators exhibit a strong innate attraction to volatiles from maize plants infested with *Z. scutellaris* or *S. littoralis*. Previous predation experiences in the presence of HIPVs influenced the predator's odor preferences. The innate preference for plants with cell or tissue damage can be explained by the fact that these plants released far more volatiles than plants infested by the phloem-sucking *D. maidis*. Yet, a predation experience on *D. maidis*-infested plants significantly increased the choices for *D. maidis*-induced maize volatiles. After *O. majusculus* experienced L3-L4 larvae (too large to serve as prey) on *S. littoralis*-infested plants they showed reduced attraction towards these plants and an increased attraction towards *D. maidis*-infested plants. When offered young larvae *S. littoralis*, which are more suitable prey, preference towards HIPVs was similar to that of naive individuals. The HIPVs from plants infested by herbivores with distinctly different feeding strategies showed clearly distinguishable quantitative differences for (Z)-3-hexenal and (E)-2-hexenal and methyl salicylate. These compounds might serve as reliable indicators of prey presence and identity for the predator.

Key Words- *Orius spp*., *Zyginidia scutellaris*, associative learning, innate, HIPVs, maize.

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INTRODUCTION

Feeding on plants by herbivores triggers the emission of complex blends of volatile compounds (herbivore-induced plant volatiles, HIPVs). These volatiles can serve as signals for natural enemies to locate their prey (Dicke and Sabelis 1987, Turlings et al. 1990), as they can provide them with valuable information on the identity and quality of potential prey on the plants (Dicke 1999; Turlings and Wäckers, 2004; Clavijo McCormick et al. 2012). In the past two decades it has been reported that volatile blends released by plants vary widely between different combinations of plant and herbivore (De Moraes et al. 1998, Van Den Boom et al. 2004), between different herbivores on the same plant species (Turlings et al. 1998, Leitner et al. 2005, Delphia et al. 2007, Gosset et al. 2009, Hare and Sun 2011, Cai et al. 2014) and between the same herbivore on different genotypes of the same plant species (Degen et al. 2004, Glinwood et al. 2011). It remains largely unclear whether the composition of the volatile blends induced by different herbivore species differs consistently enough to indicate not only that the plants are damaged by herbivores, but also the identity of the herbivore species causing the damage (Allison and Hare 2009, Clavijo McCormick et al. 2012). Yet, several studies have shown that parasitoids are more attracted to volatiles emitted by plants under attack by their specific host than the volatiles from plants with non-hosts (De Moraes et al. 1998, Chabaane et al. 2014). These distinctive volatile profiles induced by various herbivores could be caused by different feeding modes and/or specific elicitors in the insects' oral secretions when they come in contact with the damaged plant tissue during the feeding process (Yoshinaga et al. 2010).

Under Mediterranean conditions maize stands are colonized early in the season by the leafhopper *Zyginidia scutellaris* (Herrich-Schäffer), which is the first herbivore to establish in significant numbers on the aerial part of the maize plant (Pons and Albajes 2002). Leafhopper populations may build up and reach high densities during summer in the maize fields, though direct damages are rarely of economic importance. *Z. scutellaris* is a mesophyll feeder, preferentially on the older leaves, where it causes pale stripes. In a previous study we observed a correlation between cumulative numbers per plot and season of *Orius spp*. and *Z. scutellaris* (Albajes et al. 2011). We therefore hypothesize that the early colonization of maize plants by maize leafhopper is a key factor for attraction and establishment in maize fields of generalist predators as *Orius spp*. which is the prevalent on-plant predator. *Orius spp.* preys on small insects (e.g. thrips, scales, aphids, psyllids, small caterpillars and the eggs of various insects) and mites

(Péricart 1972, Riudavets et al. 1995, Lattin 1999) and are regularly found on cereals, maize and alfalfa (Pons et al. 2005, Madeira et al. 2014), as well as on weeds, depending on plant phenology and crop management.

Generalist predators like *Orius spp.* may feed on multiple prey that are heterogeneously distributed in space and time and consequently face a challenging optimal foraging task. When prey declines to low levels, predatory arthropods switch from local searching to dispersal behavior (Symondson et al. 2002), which is also the case for *O. majusculus* (Reuter) (Montserrat et al. 2004). To locate their prey in an environment with numerous potential host plants and prey, the predators rely on both their innate olfactory and/or visual preferences and memory (Drukker et al. 2000, Takabayashi et al. 2006, Dukas 2008). The behavioral responses of natural enemies to HIPVs are known to have a genetic basis (at least for mites Margolies et al. 1997, and parasitoids Gu and Dorn 2000), but are also plastic and can be modified through associative learning (Dukas 2008). The ability to associate odors with rewards is well established for parasitoids (Papaj and Lewis 1993, Turlings et al. 1993, Vet et al. 1995). This has been much less studied for predatory arthropods (see Drukker et al. 2001, Deboer et al. 2005, Glinwood et al. 2011, Lins et al. 2014).

In this paper we aim to determine whether our field observations of *Orius spp*. recruitment into maize fields reflect a specific attraction of the predators to HIPVs from *Z. scutellaris*-infested plants, and whether such attraction is affected by associative learning during prey encounters. To test this, we examined: (1) the innate preferences of *Orius majusculus* towards maize plants attacked by three herbivores with different feeding strategies, the leafhopper *Z. scutellaris* (mesophyll feeder), the leafhopper *Dalbulus maidis* (Delong y Wolcott) (phloem feeder), and the lepidopteran *Spodoptera littoralis* (Boisduval) (chewer); (2) the potential preference change in the case of previous prey experience on infested maize plants and the nature of this experience (rewarding/no-rewarding); and (3) the volatile profiles emitted by maize plants infested by the three herbivores.

MATERIAL AND METHODS

Plants and Insects

Maize seeds of the variety Delprim were sown in commercial soil (Ricoter Aussaaterde) in individual bottom-pierced plastic pots (ø 4 cm, 11 cm high). Plants were grown under natural light conditions (16:8 h L: D) in a greenhouse (24±5℃) and were watered as needed.

All insects were reared under controlled conditions (16:8 h L:D, 24±5 ºC) at the Université de Neuchâtel (Switzerland). A colony of the leafhopper *Z. scutellaris* was obtained from cereal fields at the Universitat de Lleida (Spain) and *D. maidis* was established from individuals provided by Dr. J. Bernal from a greenhouse colony maintained at Texas A&M (USA). Both colonies were reared on maize (varieties Delprim and B73). Eggs of *S. littoralis* were provided by Syngenta (Stein, Switzerland), and larvae were reared on wheat germ based artificial diet (Turlings et al. 2004).

The predator *O. majusculus* came from an established colony at the Universitat de Lleida, which is renewed every year with new individuals collected in maize fields. The colony was fed with frozen eggs of *Ephestia kuehniella* (Biotop S.A., France) as prey and green beans (*Phaseolus vulgaris L.*) as egg-laying substrate. Predators were reared in the absence of plant HIPVs. Females of *O. majusculus* were used in all experiments when they were more than a week old. In the innate preference bioassay "naïve" females coming directly from the rearing were used (12 per replicate), for the learning bioassay both "naïve" and "experienced" predators were used (12 per experience group). The day before the experiment each predator was placed individually in a 1,5mL eppendorf tube and provided with water by means of a wet cotton ball.

Odor Sources

Maize plants that were used for the experiments had 3 fully developed leaves. A day before the experiments plants were enclosed in glass bottles and infested with adults of *Z. scutellaris*, adults of *D. maidis*, or larvae of *S. littoralis*. Ten adult leafhoppers were freely released in the glass bottle with the help of an aspirator for both *Z. scutellaris* and *D. maidis* treatments. To infest plants with *S. littoralis*, five second instar larvae were transferred with a brush to maize leaves. After infestation, the bottles were maintained at laboratory temperature with a L16:D8 light cycle. The glass bottles were attached to the olfactometer setup (see Turlings et al., 2004).

Innate Prey Preference Bioassay

To test *O. majusculus* preference for *Z. scutellaris* as prey on maize we tested its attractiveness in a 4-arm olfactometer (for details see D'Alessandro and Turlings 2005) in a choice situation with a plant infested by each of the two other potential prey. In a first experiment, we tested *Z. scutellaris*-infested plants against *S. littoralis*-infested plants (n=7). In the second experiment we tested *Z. scutellaris*-infested plants against *D. maidis* (n=7). In both experiments we included an unharmed plant and an empty bottle as

127 controls. The position of the odor sources was randomly assigned each experimental day to avoid any
128 position-bias.
129 Purified and humidified air entered each odor source bottle at 1.2 l/min (adjusted by a manifold with four
130 flowmeters, Analytical Research System, Gainesville, FL, USA) via Teflon tubing and carried the
131 volatiles to the olfactometer compartment. Half of the air (0.6 l/min/olfactometer arm) was pulled out via
132 volatile collection filters that were attached to the top of each odor source bottle (see "Collection and
133 analyses of HIPVs"). These traps were connected to a vacuum pump via Tygon tubing and flow meters,
134 and airflows were balanced with a pressure gauge.
135 Half an hour before an experiment started, Eppendorf tubes containing *O. majusculus* females were
136 placed in a polystyrene box containing a plastic cooling block. In preliminary tests (not shown) we saw
137 that this cooling pre-treatment suppressed the activity of the insects and as a consequence they were more
138 receptive to odor sources and less likely to choose randomly. We adapted the olfactometer to the behavior
139 of the predator by turning the central release arena upside-down (see design in D'Alessandro and Turlings
140 2005) so insects would orient downwards, escaping the light, towards the arms of the olfactometer. We
141 released insects one by one and gave them 20 minutes to make a choice. When an insect entered an arm
142 and reached the screw cap fitting we considered it to have made a choice. Twelve females were tested per
143 replicate. The experiment was performed 7 times on different days. This resulted in 7 independent
144 replicates. All olfactometer tests were conducted between 10 am and 4pm.
145 *Predator Experience and Learning Bioassay*
146 Two series of assays were conducted to test the influence of learning on *O. majusculus* preference. In the
147 first series we evaluated the preference of *O. majusculus* when experienced to three herbivores with
148 distinct feeding modes. In the second series we evaluated the response of the predator when experienced
149 on prey, *S. littoralis* larvae, which we hypothesize that can provide both positive and negative experiences
150 depending on their developmental stage (size). Small larvae (first instar) can be readily preyed upon by
151 the minute predator, signifying a positive experience, whereas encounters with aggressive larger larvae
152 could constitute a negative experience.
153 To provide the predators with odor experiences the following procedure was used. For the three herbivore
154 bioassay on day one, 80 predators were individually placed in eppendorf tubes of 1mL, and plants were
155 enclosed one-liter plastic (PET) bottles and exposed to one of the three herbivores *Z. scutellaris, D.*
156 *maidis* or *S. littoralis* in the same density as in the olfactometer odor sources (2 plants per treatment). The

following day (day two), additional prey of each of the herbivore treatments were added to each bottle. The extra prey consisted of either 25 nymphs for the leafhopper treatments or 20 second to third instar *S. littoralis* larvae. The predators were split in four groups of experience. The first three groups were transferred into the bottles of each of the herbivore treatments. A first group was transferred to the plants infested by *Z. scutellaris* (Zs experience), the second to *D. maidis*-infested plants (Dm experience), and the third group to S. *littoralis*-infested plants (Sl experience). The fourth group of predators served as the control (control experience) with insects that were placed in two plastic cages containing *E. kuehniella* eggs and a bean pod.

The same procedure was used to examine the importance of *S. littoralis* size (developmental stage) in affecting *O. majusculus* responses after the associative experiences. Based on the *Predation bioassay*, we hypothesized that preying on young larvae (L1-L2) would constitute a rewarding experience to *O. majusculus* and that older larvae (L3-L4) would constitute an unrewarding experience. Consequently, we experienced *O. majusculus* females with L1-L2 larvae (Sl-s) and L3-L4 larvae (Sl-B) following the procedure described earlier for the three herbivores with different feeding modes. In this case the additional prey added to the odor sources consisted of thirty L1-L2 larvae for the small larvae experience and eight L3-L4 larvae for the large larvae experience. A control group was also included (control experience).

The day before the experiment (day three), each predator was again placed in a 1,5 mL Eppendorf tube and provided with water by means of a wet cotton ball. Half an hour before an experiment started, predators were placed in groups of 6 according to experience group (2 Eppendorf tubes per experience group, 12 insects in total) and placed in a polystyrene box containing a plastic cooling block.

We tested Zs, Sl, Dm and empty odor sources for both learning bioassays. As in the innate bioassay the position of the odor sources was randomly assigned for each experimental run to avoid position-bias and we used the release arena of the olfactometer upside-down. We released insects in groups of six and gave them up to thirty minutes to make a choice. On each experimental day there were two releases per experience group, testing a total of twelve females experienced with the same herbivore/treatment for each olfactometer set-up. Once we had tested the first release of all the experience groups we rotated the olfactometer 90º and then tested the second release for all treatments. The order in which we tested the different experience groups was random. Again, when an insect entered an arm and reached the screw cap fitting we considered it to have made a choice. The experiment was performed 7 times on different days

for the three herbivore learning bioassay and 8 times for the *S. littoralis* learning bioassay. Each of these days was considered as an independent replicate.

Predation Bioassay

A predation bioassay was conducted in order to evaluate the performance of O. *majusculus* on each of the offered prey. Arenas made of petri dishes (5cm in diameter) were used in the experiment. Each petri dish contained a filter paper moistened with water on which we placed a piece of maize leaf of approximately 4 cm of length. Prey corresponding to experience groups (see above) were added to the arena in groups of five. We tested four treatments: (1) *Z. scutellaris* and (2) *D. maidis* nymphs of 2^{nd} to 4^{th} stage (3) *S. littoralis* L2 instar larvae fed on maize leaves and (4) *S. littoralis* L3-L4 instar larvae fed on maize leaves. Thirty minutes later we introduced an *O. majusculus* female in each dish that had been starved for 24h, and left them for 24h. The next day we counted the number of killed prey in each of the arenas. We differentiated killed prey by *O. majusculus* females from missing prey. We compared the number of dead prey with those in control dishes without a predator. We performed the experiment two times with 8 replicates for each treatment.

Collection and Analysis of Volatiles

We collected volatiles of each odor source during the learning bioassays in the olfactometer, using adsorbent traps consisting of a glass tube (4 mm ID) packed with 25 mg Super-Q polymer (80–100 mesh) (Alltech Associates, Deerfield, Illinois, USA) for 5 hours. Each trap was attached horizontally to the top of an odor source bottle via a screw-cap outlet and connected via Tygon tubing to a flowmeter (Analytical Research System) and a vacuum pump. Air was pulled through each trap at a rate of 0.6 l/min for 5h, during each behavioral bioassay. Afterwards, the traps were extracted with 150 µl dichloromethane (Suprasolv, Merck, Dietikon, Switzerland), and 200 ng of n-octane and n-nonyl acetate (Sigma, Buchs, Switzerland) in 10 µl dichloromethane were added to the samples as internal standards. All extracts were stored at -80°C until analyses. Traps were washed with 3 ml dichloromethane before they were re-used for a next collection. Volatiles were identified with a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectometer (Agilent 5975C VL MSD). A 2-µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness; Agilent J&W Scientific, USA). Helium was used as carrier gas at constant pressure (15 psi). After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 200°C at 5°C per min followed by a post-run of 5 min at 250°C.

Chemstation software was used to estimate the quantities of all major components by comparison of the peak areas of each volatiles to the peak areas the internal standards. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 05 library and by comparison of retention times with those from previous analyses.

Statistical Analysis

We analyzed data from innate preference bioassays with a generalized linear model (GLM) with a Poisson distribution, where the number of choices by *O. majusculus* females per replicate was the response variable, and the plant odor sources and the replicate and their interaction the explicative variables. A GLM with a Poisson distribution was also used for the learning bioassays' analyses. A global analysis was performed where the response variable was the number of *O. majusculus* females per arm, experience group and replicate; the explicative variables were treatment (odor sources), experience group, replicate, and their interactions. Next, we performed an individual analysis for each of the odor sources (Dm, Sl, Zs, empty) in order to test differences between the frequencies of choice by the four/three experience groups. We considered a response to be learned when we detected a change in the choice of odor sources in experienced insects respective of the control (naïve) insects.

The proportion of *O. majusculus* females that fed on the offered prey or not in the predation bioassay were analyzed by a GLM with a Binomial distribution, with treatment, experiment and their interactions as explicative variables. As experimental day and the interaction were non-significant, they were removed from the final model. The number of prey eaten by *O. majusculus* females per treatment was analyzed with a GLM with a Poisson distribution; in this case the interaction was non-significant and was removed from the final model.

The amounts of plant volatiles were analyzed in two different ways. Firstly, we compared the amounts for each compound among treatments using a nonparametric Kruskal-Wallis test followed by Dunn’s test and adjusting p-values for multiple pairwise comparisons with the Bonferroni correction. When compounds were not detected in a treatment, analyses were performed excluding that treatment. Secondly, PLS-DA was used to determine whether samples belonging to specific herbivore treatments could be separated based on qualitative and quantitative differences in volatile emissions. The array of HIPVs may be composed of a large number of compounds and should be properly considered as an inter-correlated, multivariate suite of traits (Hare 2011). Many of these compounds share common precursors and in some cases, particular ratios of several compounds can be the product of a single enzyme. One example is

terpene synthase TPS10 in maize that forms (E)-β-farnesene, (E)- α-bergamotene, and other herbivory-induced sesquiterpene hydrocarbons from the substrate farnesyl diphosphate (Schnee et al. 2006). As a consequence compounds do no vary independently, and multivariate statistics that take into account the patterns of correlations of variables are required to determine statistically significant variation (van Dam and Poppy 2008, Hare 2011). The number of the model components was assessed graphically by checking plots of the error rate and the proportion of intergroup variance explained relative of the number of PLS components. Statistical significance of the obtained PLS-DA model was determined by m-fold cross-validation (m=7) and 999 permutations. An error rate value (%) was calculated to measure the accuracy of the classification by averaging the number of misclassifications (NMC) from each round of the cross-validation. The results of the PLS-DA analysis were represented in score plots, which reveal the sample structure according to the model components, and loading plots, displaying the contribution of the volatile emission to these components. Volatile compounds were subsequently ranked according to their respective variable importance of projection (VIP) score. The highest VIP scores reflect the relatively important contribution of compounds to the discrimination between groups. Data were log-transformed, mean-centered, and scaled to unit variance before they were subjected to the analysis. PLS-DA analysis and validation was performed using mixOmics (González et al., 2011) and RVAideMemoire (Hervé, 2014) packages. All statistical analyses were performed using R (R Development Core Team 2005).

RESULTS

Innate Behavior

We first tested the innate attraction of *O. majusculus* to the volatile blend emitted by plants infested with *Z. scutellaris*, relative to the attraction to volatiles from plants with any of the alternative prey or clean maize plants. *O. majusculus* females were attracted to *Z. scutellaris* infested plants, but when offered simultaneously, they did not distinguish between *Z. scutellaris*-infested plants and *S. littoralis*-infested plants (Fig. 1a, choice χ^2_3= 17.84, P< 0.001; replicate χ^2_1= 16.57, P=0.26; interaction χ^2_3= 15.98, P=0.90). However, *O. majusculus* markedly preferred Z. *scutellaris*-damaged plants when paired with *D. maidis*-damaged plants (Fig. 1b, choice χ^2_3= 17.12, P< 0.001; replicate χ^2_1= 16.94, P=0.68; interaction χ^2_3= 11.68, P=0.15). *D. maidis*-infested plants were as unattractive as uninfested plants or clean air (empty arm).

Learned Behavior

We also tested the effect of a previous prey experience on predator odor preferences when offered the "experienced" prey infested-plant and two alternative prey-infested plants as odor sources. *O. majusculus* females were given an experience by placing them on maize plants with *Z. scutellaris* (Zs), *S. littoralis* (Sl), or *D. maidis* (Dm), or providing them with a diet of only insect eggs without a plant (control, C). The prey-host plant experiences affected the predator's choices for the *D. maidis* odor source, but not the choices for the other two infested plant types (Fig. 2). This was reflected in a significant effect of the type of experience and the interaction term (choice × experience) in the model (interaction χ^2_9= 130.4, P=0.02). Compared with control predators, the number of choices for Dm was increased by two-thirds in Dm-experienced individuals (experience χ^2_3= 25.17, P=0.023; replicate χ^2_6= 20.61, P=0.60; interaction χ^2_{18}= 14.85, P=1; Fig. 2). Interestingly, this increase in preference for Dm was also observed for Sl-experienced predators, whereas Zs-experienced predators showed an increased tendency to avoid Dm in favor of the Zs treatment (Fig. 2).

Females of *O. majusculus* can experience *S. littoralis* prey positively or negatively depending on the larval instar encountered (see Results: *Predation on offered prey*), and their subsequent responses are affected accordingly (significant choice × experience term in the model χ^2_8= 178.15, P<0.01, Fig. 3). When experiencing a rewarding predation on *S. littoralis* (Sl-s), predator preference for odor treatments was similar to that of the predators from the control. In contrast, after facing an unrewarding experience on large larvae (Sl-B) *O. majusculus* were less attracted to the odor *S. littoralis*-infested plants (experience χ^2_2= 32.71, P<0.01; replicate χ^2_7= 23.50, P=0.24; interaction χ^2_{14}= 14.14, P=0.81) and tended to be more attracted to *D. maidis*-infested plants (Fig. 3), similar to what was found during the first learning bioassay. The proportion of females that did not choose was also similar for both bioassays.

Predation on Offered Prey

We performed a predation acceptance experiment to estimate the preference of *O. majusculus* females for the different prey offered in the learning bioassays. Predators fed on all prey offered (pie chart in Fig. 4), but the proportion of females that fed differed considerably between treatments (χ^2_3= 67.5, P< 0.001). Almost all predators that were offered small *S. littoralis* or *Z. scutellaris* fed on these prey, but only a small fraction of the predators managed to consume one of the large *S. littoralis* larvae. Overall, there were clear differences in the number of prey killed by females after 24h (experiment χ^2_1= 4.60, P=0.03; treatment χ^2_3= 102.9, P< 0.001; Fig 4). Predators were most successful feeding on small larvae and *Z. scutellaris* nymphs, followed by *D. maidis* nymphs and large *S. littoralis* larvae (Fig. 4). The large

differences in consumption of small and large *S. littoralis* by *O. majusculus* females, are likely to reflect rewarding and non-rewarding experiences respectively, as is evident from their subsequent responses to the odor sources.

Volatile Profiles

Volatile blends from plants attacked by the herbivores *Z. scutellaris*, *D. maidis* and *S. littoralis* from the first learning experiment were analyzed. Twenty compounds were identified from previous studies (Table 1) (Turlings et al. 1998, Degen et al. 2004, Erb et al. 2010), plus an unknown compound also detected by Turlings et al. (1998), probably a nitrogen containing compound present also in healthy plants. Twenty of those volatile compounds were quantified (Table 1). A PLS-DA analysis of volatiles emitted by plants infested with *Z. scutellaris*, *S. littoralis* and *D. maidis* showed two significant principal components (PLS), explaining 72.12 and 7.5 % of the total variance, respectively (Fig.5). The error rate value (%) calculated by permutation was < 3% ($p=0.001$). The first component (PLS1) separated the volatile blends based on the amount of emitted volatiles caused by the feeding of each of the three herbivores, exposing the quantitative differences in emission rates. The second component (PLS2) separated blends qualitatively according to the presence or absence of certain compounds or a difference in their proportions in the total blend. These discriminating compounds were the three that had a VIP value higher than 1 (Table 1). In decreasing order of importance, the compounds were the green leaf volatiles (GLVs) (Z)-3-hexenal and (E)-2 hexenal, and methyl salicylate (Table 1, Fig. 5). Globally, *Z. scutellaris* treated plants emitted the largest amounts of volatiles, followed by *S. littoralis* plants, whereas *D. maidis* plants emitted the smallest amounts and number of volatile compounds (Table 1, Fig. 5). Unlike *S. littoralis*, neither *Z. scutellaris* nor *D. maidis* feeding resulted in detectable release of (Z)-3-hexenal and (E)-2 hexenal. On the other hand methyl salicylate was detected in both Sl and Zs treatments, but its proportion was highest for Zs infested plants (Table 1, Fig. 5).

DISCUSSION

Innate preferences and associative learning

We found that the anthocorid predator *O. majusculus* has an innate preference for *Z. scutellaris*- and *S. littoralis*-induced volatiles, and that this preference can be modified through associative learning. The innate preference suggests that the anthocorid predator is initially mainly attracted to volatiles that result from tissue and/or cell damage, as opposed to volatiles that are emitted in response to phloem feeding.

336 This changed when they successfully fed on nymphs of the phloem feeder *D. maidis*. After preying on *D.*
337 *maidis* nymphs on *D. maidis*-infested plants, the predator's preference shifted towards *D. maidis*-induced
338 volatiles.
339 By contrast, the predator's odor preferences after a feeding experience on *S. littoralis* larvae depended on
340 the developmental stage of the prey larvae. A reduced attractiveness towards *S. littoralis*-infested plants
341 was observed when predators were experienced on large larvae. This can be explained by a possible
342 negative association of the feeding experience (the larvae were too large for consumption) with the plant
343 odor. After the predators were placed with small larvae, which can be considered a positive experience,
344 their odor preferences did not differ from those of naïve predators.
345 Learning of HIPVs by *O. majusculus* was expected, as it has been frequently observed in generalist
346 carnivores (Steidle and Van Loon 2003). Intriguingly, just as the discriminant analysis could separate
347 volatile blends emitted by maize plants attacked by the three herbivores, the predators appear to be able to
348 do the same. They appear to use this ability to discriminate between the odor blends in order to focus their
349 foraging efforts on the most profitable odor source. Overall, a positive feeding experience resulted in or
350 maintained a preference for the odor that was associated with this positive experience, whereas a negative
351 experience (large *S. littoralis* larvae) reduced the response to the experienced odor. The predator feeding
352 experiment revealed clear differences in the suitability of small and large *Spodoptera* larvae as prey (Fig.
353 4). This might be explained by prey quality (but see Venzon et al. 2002) and by differences in handling
354 time and/or prey's aggressive and escape behavior (Heady and Nault 1985, Montserrat et al. 2000,
355 Eubanks and Denno 2000). The flexibility in the predator's foraging behavior might facilitate its dispersal
356 to plants where it will find prey and be more effective in controlling pests. The effects of negative
357 associations are likely to quickly diminish upon dispersal, as it has been proposed for parasitoids (Papaj
358 1994, Takabayashi et al. 2006). Moreover, *O. majusculus* ability to learn by association is good news in
359 the context of colonization by non-native pests, as potentially the predatory community could adapt to
360 new prey species, also if they have entirely different feeding habits.
361 *Feeding strategies and HIPVs profiles*
362 Plant responses to herbivore attack can strongly depend on the herbivore's feeding strategy and the
363 amount of tissue damage occurring at the feeding site (Walling 2000). For chewing herbivores, it is well
364 established that plant damage together with salivary enzymes, such as glucose oxidase, and non-
365 enzymatic elicitors present in the oral secretions can trigger the release of plant volatiles (Alborn et al.

1997, Musser et al. 2002; Schmelz et al., 2006). A potent elicitor in the oral secretions from *Spodoptera* and other caterpillars, the fatty acid-amino acid conjugate elicitor volicitin, induces a systemic release of volatiles from maize plants (Alborn et al. 1997, Yoshinaga et al. 2010) acting via the jasmonate pathway (Schmelz et al. 2003). Two other types of elicitors are known, inceptins (disulfide-bridged peptide in *S. frugipeda* (Smith)) (Schmelz et al. 2006) and caeliferins (saturated and monounsaturated sulfated α-hydroxy fatty acidsin the grasshopper *Schistocerca americana* (Drury)) (Alborn et al. 2007).

Considerably less is known about the molecular mechanisms implicated in the differential plant defense responses to mesophyll and phloem-feeding insects. Most typhlocybine leafhoppers like *Z. scutellaris* feed using a sawing laceration strategy, leaving round, silvery-white marks called stipples (Marion-Poll et al. 1987, Backus et al. 2005). Phloem feeding insects, like *D. maidis*, form stylet-sheaths following intercellular (Sternorryncha, e.g. aphids) or intracellular (Auchenorryncha e.g. *D. maidis*) sucking pathways (Backus et al. 2005). Salivary enzymes and elicitors for Auchenorrhyncha are not well studied and it can only be inferred that cell degrading enzymes similar to those found in Thysanoptera (Stafford-Banks et al. 2014) or Heteroptera (reviewed by Sharma et al. 2014) play a critical role in their feeding behavior. Recently there was the first attempt to characterize salivary glands in leafhoppers, and a transcriptome of the salivary glands of the mesophyll feeder *Empoasca fabae*'s revealed the presence of enzymes such as amylases, lipases and trypsin, and detoxifying enzymes such as superoxide dismutase (DeLay et al. 2012). A role of these compounds in plant defense responses and volatile emissions remains to be determined.

The discriminant analysis on herbivore-induced volatile blends shows that the plant's response to insects with distinctly different feeding strategies can be distinguished quantitatively (PLS1) and by discriminating compounds on the other (e.g. GLVs). Notably, mesophyll feeding *Z. scutellaris* induced volatile profiles that resembled the ones induced by the chewer *S. littoralis*, suggesting that the induction of plant volatile by *Z. scutellaris* adults can be as strong as caterpillars on a per capita basis. On the other hand, phloem feeding *D. maidis* induced only few volatiles (seven out of twenty-one detected), which were released in considerably smaller amounts. Hence, *Orius spp.* preference for maize plants damaged by a chewer and a mesophyll feeder can be explained by the fact that these plants released far more volatiles than *D. maidis* infested plants.

(Z)-3-hexenal and (E)-2 hexenal together with methyl salicylate were the discriminating compounds to distinguish the volatile profiles of the three herbivores (Fig. 5). These GLVs are cell wall breakdown

products and commonly found to be released by plants under attack by chewing insects, but here they were not detected for either of the two leafhopper treatments. The lack in the emission of these GLVs has also been reported in maize for phloem-feeding aphids (Turlings et al. 1998) and the leafhoppers *Euscelidius variegatus* (Erb et al. 2010) and *Cicadulina storeyi* (Oluwafemi et al. 2011). Interestingly, the reported overall volatile emission and number of detected compounds for *E. variegatus* and *C. storeyi* infested plants was much larger than the one observed for *D. maidis.* This could be explained by either the density of insects used for plant induction, thirty for *E. variegatus* and fifty *C. storeyi*, versus ten for *D. maidis*, but more likely by the type of damage inflicted by the phloem feeding insects or the elicitors that are implicated in the induction (Sharma et al. 2014). *D. maidis* is a specialist on maize and its wild ancestor teosinte, and it may have evolved ways to avoid or suppress defense responses in these plants (Nault and DeLong 1980, Dávila et al. 2013).

Methyl salicylate is one of the compounds that seems to be of particular importance in mediating attraction of several natural enemies, and a recent meta-analyses concluded that it acts as a broad spectrum attractant (Rodriguez-Saona et al. 2011 and references therein). Predatory taxa like *Orius spp*., Chrysopidae, Syrphidae, and Coccinellidae among others are attracted to synthetic methyl salicylate when deployed in the field (James and Price 2004, Zhu and Park 2005, Mallinger et al. 2011). We detected methyl salicylate at high levels in *Z. scutellaris*-induced plants, reinforcing our hypotheses that *Z. scutellaris* mediated recruitment of generalist natural enemies into maize fields. *Z. scutellaris* colonizes maize fields early in the season and they may reach up to 100 individuals per plant before pollen shed (Pons and Albajes 2002). At this early stage, colonization by key Lepidopteran pests like *Sesamia nonagrioides* (Lefebvre) and *O. nubilalis*, and occasional pests like *Helicoverpa armigera* (Hübner), *Mythimna unipuncta* (Haworth) and *Spodoptera* spp. is low (Pons and Albajes 2002). These pests will arrive later and the presence of *Orius spp.*, thanks to early infestation by *Z. scutellaris*, may greatly reduce the negative impact of the Lepidopteran pests.

Conclusions

In summary, the results show that generalist insect predator *O. majusculus* is attracted to herbivore-induced plant volatiles and that its responses to these volatiles are flexible and affected by positive and negative experiences during prey encounters. The innate preference for volatiles released upon infestation by *Z. scutellaris* and *S. littoralis*, can be explained by the fact that these insects damage cause cell tissue damage, resulting in far larger amounts of volatiles than released from infested plants by the phloem

feeder. Yet, the innate preference can be modified in favor of normally less preferred HIPVs after a rewarding experience with prey. Three compounds, (Z)-3-hexenal and (E)-2-hexenal and methyl salicylate were found to be most predictive in indicating whom was feeding on a plant and might be used by the predators to discriminate between plants with potential prey. Taken together, the results support the notion that feeding by *Z. scutellaris* results in the emission of maize's HIPVs that initially recruit of *Orius spp*. into maize fields.

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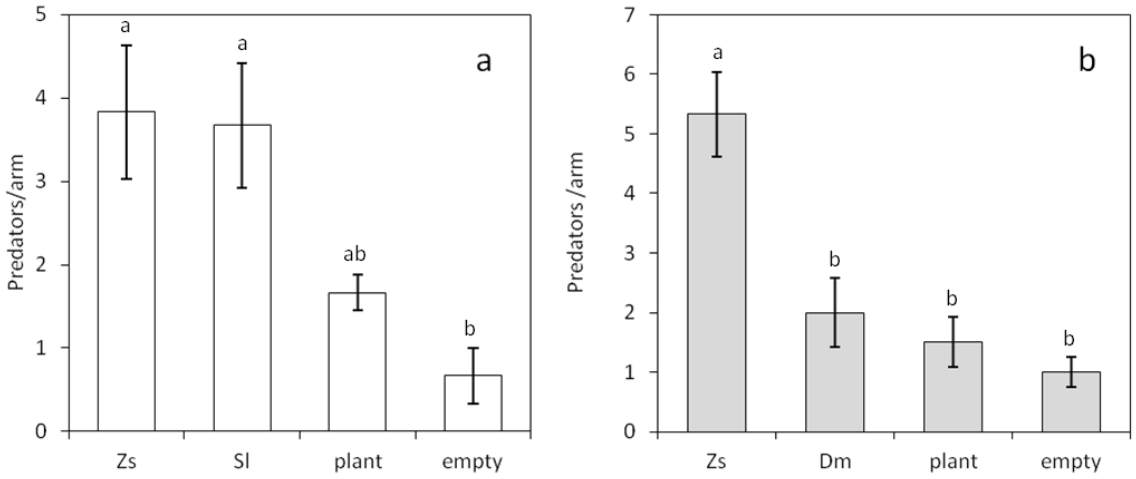

Fig. 1 Choices for herbivore-induced plant odors by *O. majusculus* shown as the average numbers (±SE) per trial. In a first experiment (a) *Z. scutellaris* vs. *S. littoralis* herbivore-induced plant odors were tested (n=7); in a second (b) *Z. scutellaris* vs. *D. maidis* herbivore-induced plant odors were tested (n=7). Odor sources Dm= *D. maidis*-damaged plant; Sl= *S. littoralis*-damaged plant; Zs= *Z. scutellaris*-damaged plant; Plant= maize plant; empty = empty arm. Different letters indicate significant differences between treatments ($p < 0.05$).

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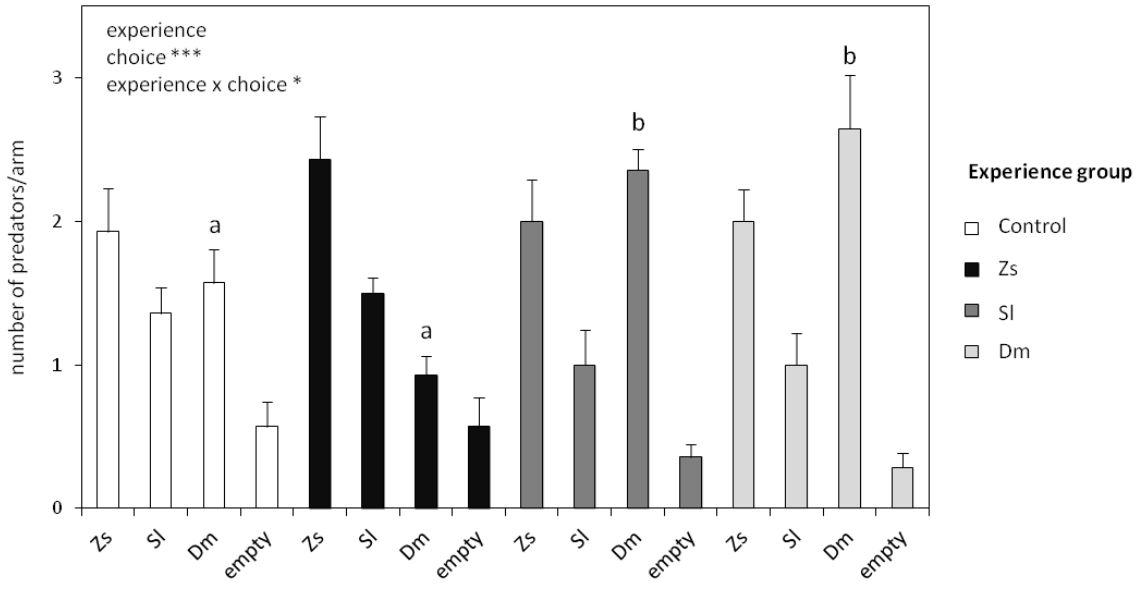

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612 Fig 2 Choices for herbivore-induced plant odors by *O. majusculus* females with
613 different previous prey-experiences, shown as the average numbers (+SE) of predators
614 per release group of six. Four odor sources were tested Dm = *D. maidis*-damaged plant;
615 Sl= *S. littoralis*-damaged plant; Zs= *Z. scutellaris*-damaged plant; empty = empty arm.
616 Prey experience was provided on infested plants with extra prey of three herbivores
617 Dm= *D. maidis*; Sl= *S. littoralis*; Zs= *Z. scutellaris*; and a Control with only *E.*
618 *kuheniella* eggs (in the absence of a plant). Different letters indicate significant
619 differences between the control experience group (naïve insects) and other prey
620 experience groups ($p < 0.05$). * indicates a significant interaction ($p<0.05$).

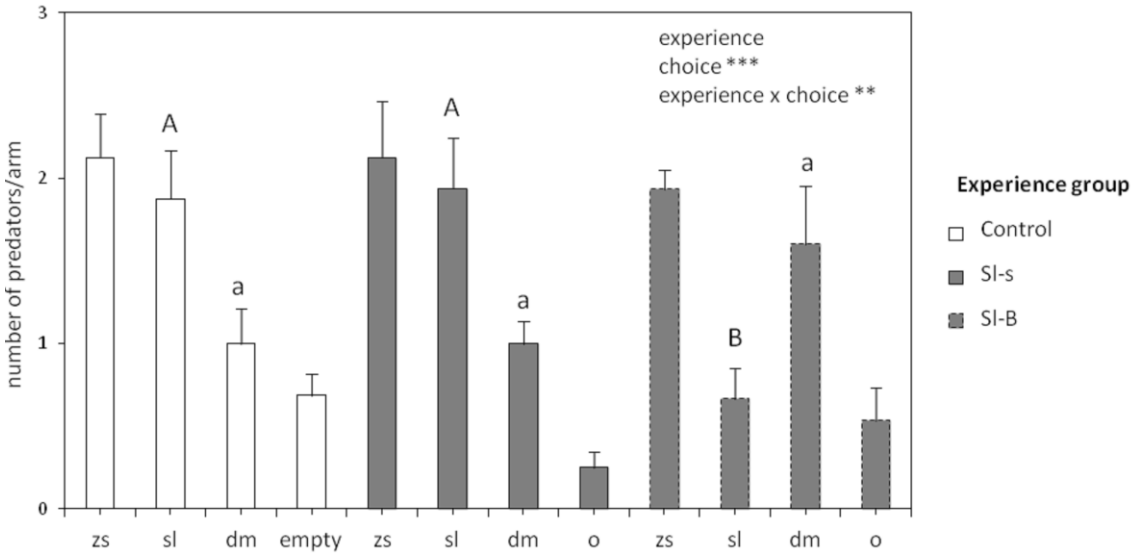
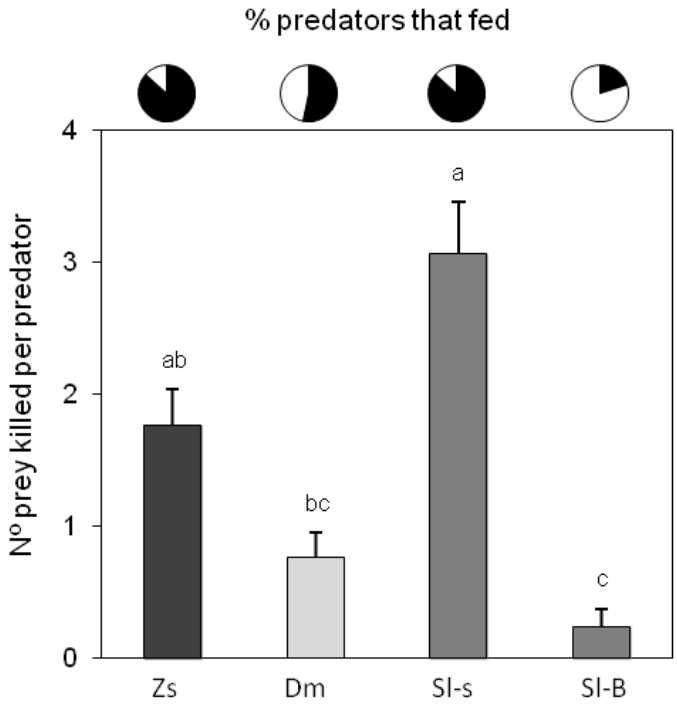


Fig. 3 Choices for herbivore-induced plant odors by *O. majusculus* females with different previous prey-experiences, shown as the average numbers (+SE) of predators per release group of six. Four odor sources were tested Dm = *D. maidis*-damaged plant; Sl= *S. littoralis*-damaged plant; Zs= *Z. scutellaris*-damaged plant; empty = empty arm. Prey experience was provided on *S. littoralis* infested plants of two sizes Sl-s= small and Sl-B= big, and a Control with only *E. kuheniella* eggs (in the absence of a plant). Different letters indicate significant differences in the odor choice between the control experience group (naïve insects) and other prey experience groups ($p < 0.05$). Uppercase letters indicate differences in the response to the Sl odor source; lowercase letters indicate differences in the response to the Dm odor source. ** indicates a significant interaction ($p<0.01$).



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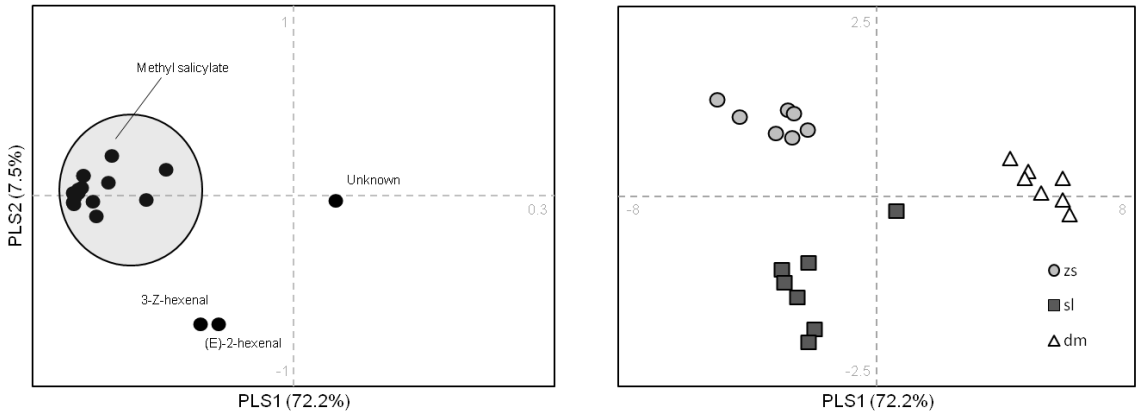
634 Fig. 4 Proportion of *O. majusculus* females that fed on offered prey during 24h (black
635 proportion in pie charts), and average number of herbivorous prey eaten by the
636 predators (bar graph) shown as the average (+SE). Six treatments were offered: Zs= 2nd
637 to 4th instar *Z. scutellaris*; Dm= 2nd to 4th instar *D. maidis*; Sl-s= 1st to 2nd instar *S.*
638 *littoralis*; Sl L3-B= 3rd to 4th instar *S. littoralis*. Different letters indicate significant
639 differences between treatments ($p < 0.05$).



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641 Fig. 5 Loading (a) and score (b) plots for the two components of the PLS-DA used to
642 discriminate between volatile blends emitted by plants infested by *Z. scutellaris* (Zs), *S.*
643 *littoralis* (Sl) and *D. maidis* (Dm).

644 Table.1 Volatile emissions (ng/h) of plants infested by *Z. scutellaris*, *S. littoralis* or *D.*
645 *maidis* and variable influence on projection (VIP) values for each compound for the
646 PLS-DA model. Amounts of each compound were compared among treatments using a
647 non-parametric Kruskal-Wallis test followed by Dunn's test and adjusting p-values for
648 multiple pairwise comparisons with the Bonferroni correction (*p <0.05, **p < 0.01,
649 ***p < 0.001). Compounds denoted with "N" were only tentatively identified by
650 comparison of their MS to that reported in libraries. In bold compounds with VIP>1.
651 n.d. not detected, d detected in a small fraction of samples.

		Z. scutellaris		*S. littoralis*		*D. maidis*			
	VIP	**ng/h**	**±SE**	**ng/h**	**±SE**	**ng/h**	**±SE**	χ^2	**P**
1. Unknown	0.16	3.02	0.11	3.10	0.13	3.39	0.14	1.21	n.s
2. (Z)-3-hexenal	**2.27**	**n.d**		**3.37**	**0.35**	**n.d**			
3. (E)-2-hexenal	**1.97**	**n.d**		**3.02**	**0.37**	**n.d**			
4. β-myrcene	0.52	3.78a	0.39	2.86a	0.18	0.83b	0.11	11.96	*
5. Z-3-hexenyl acetate	0.82	13.39	1.97	4.75	0.50	n.d		3.92	*
6. (Z)-β-ocimene	0.74	1.06	0.16	0.80	0.08	n.d		0.15	n.s
7. Linalool	0.77	100.97a	8.06	72.38a	3.78	19.51b	1.59	13.30	*
8. DMNT	0.68	45.22a	6.08	15.88a	1.27	0.92b	0.12	16.20	***
9. Phenyl-methyl acetate	0.82	6.98	1.68	1.79	0.23	n.d		3.43	·
10. Methyl salicylate	**1.07**	**2.01**	**0.29**	**0.49**	**0.08**	**n.d**		**4.48**	*****
11. 2-phenethyl acetate	0.83	4.19	0.58	1.40	0.15	n.d		3.92	*
12. Indole	0.89	102.57	14.40	43.30	2.54	n.d		1.47	n.s
13. Methyl anthranilate		d(3/7)		d(1/7)		n.d			
14. (E)-geranyl acetate	0.88	29.26	3.68	11.99	0.90	n.d		3.43	·
15. E-β-caryophyllene	0.80	22.98	5.02	6.72	0.54	d(2/7)		0.33	n.s
16. (E)-β-bergamotene	0.83	230.83a	29.98	81.39a	3.27	6.88b	1.22	15.15	***
17. E-β-farnesene	0.83	481.11a	59.90	165.19a	7.39	12.02b	2.51	15.38	***
18. α-zingiberene^N	0.87	8.70	1.50	1.95	0.12	n.d		4.44	*
19. β -bisabolene	0.87	14.80	2.39	4.05	0.23	n.d		3.43	·
20. β -sesquiphellandrene^N	0.87	41.19	6.74	10.72	0.55	n.d		4.44	*
21. TMNT	0.87	10.71	1.77	2.26	0.13	n.d		6.21	*

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